SYNTHESIS & CHARACTERIZATION OF ZEOLITES FROM COAL FLY ASH FOR PURIFICATION OF ALPHA AMYLASES

A Thesis

submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled, **"Synthesis and characterization of zeolites (from coal fly ash) for the purification of alpha amylases"** in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology and Environment Sciences, Thapar university, Patiala, Punjab; is an authentic record of my own work during a period of six months from January 2013 to july 2013, under the supervision of Dr. Moushumi Ghosh, Associate Professor, Department of Biotechnology and Environmental Sciences, and Dr. Sanghamitra Barman, Assistant Professor, Department of Chemical Engineering, Thapar University, Patiala. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any other degree.

Place: Patiala
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CERTIFICATE

This is to certify that the thesis entitled "**Synthesis and characterization of zeolites from coal fly ash for the purification of alpha amylases**" submitted by Nitin Goyal in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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To my parents, for all the support and motivation they have given me throughout my life.....

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CONTENTS

Annexure a

ABSTRACT

The biotechnological potential of α-amylases from microorganisms has drawn a great deal of attention from various researchers worldwide as likely biological catalysts in a variety of industrial processes. Currently recovery of alpha amylase from the various culture broths represent a complex engineering problem, involving multi-step schemes that lead to significant loss of the desired bio product by conventional methods. The bio product is not only present in very low concentrations, but also it is subject to chemical/enzymatic degradation. An alternative cost-effective method for bio separation/recovery of proteins in a highly purified form may be through adsorption, which is a separation technique based on specific and reversible binding via biological reactions.

In the present study, a cheap source i.e coal fly ash was used to synthesize three types of zeolites by alkali fusion followed by hydrothermal treatment. The synthesized zeolites were characterized through XRD (X-Ray Diffraction), SEM (Scanning Electron Microscope) and FTIR (Fourier Transform Infrared Spectroscopy) techniques. The cost of production of synthesized zeolites was estimated to be almost one-fifth of that of commercial zeolites available in the market. The effective adsorption of alpha amylases from bacterial culture by newly synthesized adsorbents was studied and the recovery of alpha amylase from zeolites was performed by desorption. The recovery of α-amylase was observed to be maximum (73%) in case of zeolite NaX. The results of the present study are encouraging for the use of zeolites for purification of industrially important alpha-amylases economically.

Keywords: Adsorption, alpha amylase, characterization, coal fly ash, purification, zeolites.

LIST OF ABBREVIATIONS

AL_2O_3	Aluminium oxide
BEA	Zeolite beta
CFA	Coal fly ash
et al.	Et alteri/et alii (and others)
FAU	Faujasite type zeolite
FTIR	Fourier transform infrared spectrometry
L.lactis	*Lactococcus lactis*
LTA	Linde type A
MRS	Man Ragosa sharpe
NaOH	Sodium hydroxide
O.D	Optical density
SEM	Scanning electron microscope
SiO_2	Silicon dioxide
TEAOH	Tetraethyl ammonium hydroxide
XRD	X-ray diffraction
A	Alpha

LIST OF SYMBOLS

%	Percentage
µl	Micro litre
µm	Micrometre (1×10^{-6}m)
^{0}C	degree(s) Celsius
G	Gram
Mg	Miligram
mg/ml	Milligram per millilitre
Min	Minutes
Ml	Millitre
Rpm	Revolutions per minute
Sec	Second
U	Unit
wt%	Weight percent

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

1. INTRODUCTION

Amylolytic enzymes are of great significance in biotechnological applications ranging from food, fermentation, textile to paper industries. Although amylolytic enzymes can be derived from several sources, such as plants, animals and microorganisms, the enzymes from microbial sources generally degrade starch to meet industrial demands and had made significant contribution to the production of foods and beverages in the last three decades. The microbial amylases have almost completely replaced chemical hydrolysis of starch in starch processing industry. The estimated value of world market is presently about US$ 2.7 billion and is expected to increase by 4% annually through 2013. Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) are the main industries, which use about 75% of industrially produced enzymes. Overall amylases constitute as an industrial enzymes comprising of approximately 25% of the global enzyme market.

Alpha amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*--glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. The level of alpha amylase activity in various human body fluids is of clinical importance e.g. in diabetes, pancreatitis and cancer research, while plant and microbial alpha amylases are used as industrial enzymes. Most of the alpha amylases are metalloenzymes, which require calcium ions (Ca^{+2}) for their activity, integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes. The alpha amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying or transglycosylating enzymes. Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques.

The downstream processing of α-amylase has been investigated in several studies, since they are bio products that are not only present in very low concentrations, but also they are subject to chemical/enzymatic degradation. An alternative cost-effective method for bio separation /recovery of proteins in a highly purified form may be through adsorption, which a separation technique based on specific and reversible is binding via biological reactions (Miller *et al*., 2005). In earlier reports, α-amylase has been immobilized on zirconia or alumina via adsorption and exhibited higher activity; it was covalently immobilized on phthaloyl chloride-containing amino group functionalized glass beads forming amide bonds between amino groups on protein and acid chloride groups on the glass surface (Grutzeck and Siemer, 1997). Alpha-amylase was also immobilized in modified ordered mesoporous silicas through the reaction of free –CHO (following alkylamine and glutaraldehydeprocedures) with $–NH_2$ of enzyme for hydrolysis of starch which resulted in improved thermal and pH stability of enzyme. On account of these limitations, it is necessary to investigate the properties of high silica zeolite as an enzyme support material. In this regard, zeolites synthesized from coal fly ash may be anticipated to be a potential adsorbent for the adsorption and purification of alpha amylases.

The amount of coal fly ash (CFA) generated by coal based thermal power plants has been increased at an alarming rate throughout the world. The disposal of such a big quantity of fly ash has become an important topic. Thus, several new approaches have been adopted to utilize fly ashes not only to reduce the cost of the disposal but also to minimize environmental impact. Recently, intensive efforts were made to promote the recycling of fly ash through zeolitization. Zeolites are microporous crystalline alumina silicates with three dimensional framework structures. Due to the high thermal and good dimensional stability, they have attracted a particular attention as adsorbents. By virtue of their compositional and structural peculiarities, zeolites also have many other applications in various purification

processes, adsorption, ion-exchange and separation processes. Typical examples of zeolites that were useful in the study for retaining and purification of alpha amylases are hydrophobic zeolite, such as zeolite NaX, zeolite beta and zeolite A. In the present study, three different zeolites were synthesized from coal fly ash for comparative study of their adsorption and purification towards alpha amylases and to investigate their applicability in purification processes.

Consequently, the following objectives were framed to achieve the designed targets:

- Synthesis of zeolites from coal fly ash.
- Characterization of synthesized zeolites.
- Purification of alpha amylases by zeolites.

REVIEW OF LITERATURE

2.1 Alpha Amylase

Alpha amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*--glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the alpha amylases are metalloenzymes, which require calcium ions (Ca^{+2}) for their activity, integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes. Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry. These enzymes account for about 30% of the world's enzyme production. The alpha amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying or transglycosylating enzymes. Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques (Sivaramakrishnan *et al.*, 2011).

2.2 Discovery of amylases (Aiyer, 2005).

Early in the 19th century, many scientists studied the factors responsible for the digestion of starch in cereal extracts. Nasse (1811) found that starch extracted from living plants was capable of effecting its own conversion to sugar, but this transformation was not observed when the starch was taken from plants which have been killed by boiling water. Kirchoff (1815) performed an ingenious experiment showing gluten (or malt) responsible for formation of sugar in flour steeped in warm water, as a result of seed germination: gluten acquired a capacity to convert a much larger quantity of starch into sugar than that is

contained in the seed. Thus kirchoff laid the foundation for the discovery of a substance with the property of grain protein in converting starch into sugar.

Payen and Persoz (1833) found that an alcohol precipitate of malt extract contained a thermo-labile substance which converted starch into sugar. This substance, which is now known as AMYLASE, was named "diastase". It was the first time that the active principle was named. In 1886, Lintner reported that barley malt had two diastases (amylases): a starch liquefier and a starch saccharifier. Kuhn (1924) classified the amylolytic enzymes into two types. The saccharogenic amylase of malt was named β-amylase after its ability to hydrolyze starch to the β-anomeric form of maltose. The liquefying and dextrinizing enzymes were classified as α-amylases.

2.3 Alpha Amylase Production

Alpha amylases are one of the most popular and important form of industrial amylases and the present review highlights the various aspects of microbial alpha amylases (Gupta *et al* 2003 and Reddy *et al*., 2003).

Alpha amylases are ubiquitous in distribution. However in the past few decades, considerable research has been undertaken with the extracellular alpha amylase being produced by a wide variety of microorganisms. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics. Alpha amylase has also been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Ojano *et al.,* 2007 and Petrov *et al.,* 2008).

The history of the industrial production of enzymes dates back to the time when Dr. Jhokichi Takamine began the production of digestive enzyme preparation by wheat bran

koji culture of *Aspergillus oryzae* in 1894. Industrial production of dextrose powder and dextrose crystals from starch using alpha amylase and glucoamylase began in 1959. Since then, amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrins forms the major part of the starch processing industry (Marshall, 1975). The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products and beverages. Hydrolysis of starch to products containing glucose, maltose, etc is brought about by controlled degradation (Norman, 1978; Barfoed, 1976; Hurst, 1975).

2.3.1 Alpha amylase production from *Lactococcus lactis* by fermentation

Various *Lactobacillus* strains exhibit amylase activity*: Lb. cellobiosus, Lb. amylovorus , Lb. amylophilus* and *Lb. Amylolyticus, Lb. plantarum* and *Lb. manihotivorans* have been isolated from cassava-based fermented products. Amylolytic strains of *Lb.plantarum* and *Lb. fermentum* have been isolated from cereal-based fermented foods Amylolytic enzymes from lactic acid bacteria have several applications in the starch industry and are commercially important in the beverage, food, and textile industries (Wasko *et al*., 2010).

2.4 Applications of alpha amylases

2.4.1 Starch conversion

The most widespread applications of alpha amylases are in the starch industry, which are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and saccharification, involving the production of glucose and maltose via further

hydrolysis. Initially, the alpha amylase of *Bacillus amyloliquefaciens* was used but it has been replaced by the alpha amylase of *Bacillus stearothermophilus* or *Bacillus licheniformis*. The enzymes from the *Bacillus* species are of special interest for large-scale biotechnological processes due to their remarkable thermostability and because efficient expression systems are available for these enzymes (De Souza and Magalheas, 2010).

2.4.2 Direct fermentation of starch to ethanol

The amylolytic activity rate (Abouzied and Reddy, 1986) and amount of starch utilization and ethanol yields increase in several fold in co cultures (Van Lenen and Smith, 1968). Moulds amylases are used in alcohol production and brewing industries. The advantages of such system are uniform enzyme action in mashes, increase rate of saccharification, alcohol yield and yeast (Van Lenen, 1968).

2.4.3 Textile industry

Amylases are used in textile industry for desizing process. Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be removed quite easily. Starch is later removed from the woven fabric in a wet-process in the textile finishing industry. Desizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. The alpha amylases remove selectively the size and do not attack the fibres. It is worthwhile to mention that amylases from *Bacillus* species have been employed in textile industries for over a long time (De Souza and Magalheas, 2010).

2.4.4 Paper industry

Since, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with amylases in a batch or continuous

processes. Starch is a good sizing agent for the finishing of paper, improving the quality and erasebility, besides being a good coating for the paper. The size enhances the stiffness and strength of paper (De Souza and Magalheas, 2010).

Amylases, especially alkaline amylases are used in detergents (Aiyer, 2005). To some extent amylases are also used as digestive aids (Beazell, 1942) to supplement the diastatic activity of flour and to improve digestibility of some of the animal feed ingredients**.**

2.5 Purification of alpha amylase

Industrial enzymes produced in bulk generally require little downstream processing and hence are relatively crude preparations. The commercial use of alpha amylase generally does not require purification of the enzyme, but enzyme applications in pharmaceutical and clinical sectors require high purity amylases. The enzyme in purified form is also a prerequisite in studies of structure function relationships and biochemical properties. The purification of alpha amylases from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from the fermentation broth, selective concentration by precipitation using ammonium sulphate or organic solvents such as chilled acetone. The crude enzyme is then subjected to chromatography, usually affinity, ion exchange and/or gel filtration. In recent years, fermentation processes have become more industrially successful because of the increasing demand for naturally produced alpha amylase. The major drawback of processes was the high cost of recovery, purification, which represents a considerable portion of the alpha amylase production costs (Gupta *et al.,* 2003).

At present, protein purification is a challenge because in addition to the particular protein that is meant to be purified, the protein's cell contains several thousand other proteins along with nucleic acids (DNA and RNA), polysaccharides, lipids as well as small molecules.

By purifying a protein, a specific protein will be separated from contaminants in a manner that it will not produce a useful end product which is not a cost effective approach. An alternative cost-effective method for bio separation/recovery of proteins in a highly purified form may be through adsorption, which is a separation technique based on specific and reversible binding via biological reactions. In the present study, we aimed to develop a cost efficient method to purify alpha amylase from a bacterial culture by zeolites.

2.6 Adsorption and Binding

Adsorption occurs whenever a solid surface is exposed to a gas or liquid and it is defined as the enrichment of material or increase in the density of the fluid in the vicinity of the interface. The term adsorption may also be used to denote the process in which adsorptive molecules are transferred to, and accumulate in, the interfacial layer. Its counterpart, desorption, denotes the converse process in which the amount adsorbed decreases. Based on the nature of the bonding between the adsorbate molecule and the solid surface, adsorption can be categorized as either physical adsorption, which doesn't involve chemical bonding or chemisorption which involves chemical bonding.

The unique advantage of adsorption over other separation methods is the higher selectivity that can be achieved by adsorbents. In addition, adsorption phenomena play a vital role in many solid state reactions and biological mechanisms. There are many industrial applications of adsorption. The most significant commercial adsorption-related applications in industry are the purification of acid natural gas streams, gas drying, ammonia removal from gases, air separation and deodorization (Ruthven, 1984).

2.7 Zeolites as potential adsorbents

In the adsorption process, adsorbent is an additional component, which plays a crucial role as it is the solid material on which adsorption occurs. Commercially useful adsorbents can be classified by the nature of their structure (amorphous or crystalline), by the sizes of their pores (micropores, mesopores, and macropores), by the nature of their surfaces (polar, nonpolar or intermediate), or by their chemical composition. All of these characteristics are important in the selection of the best adsorbent for any particular application (Ruthven, 1984).

Some adsorbents are used on large scale as dessicants, catalysts or catalyst supports while others are used for the separation of gases, the purification of liquids, pollution control or for respiratory protection. There are different adsorbents being used in industry such as active carbon, silica gel, silicalites, activated clays, synthetic zeolites, natural zeolites (Clinoptilolite, Erionite, Mordenite), 4A, 5A, 13X molecular sieves and activated aluminas. For an adsorption process to be developed on a commercial scale requires the availability of a suitable adsorbent in tonnage quantities at economic cost. The earlier adsorption processes used either activated carbon or silica gel adsorbents but the potential of adsorption as a separation process was greatly enhanced by the development of molecular sieve adsorbents such as natural and synthetic zeolites (Ojha *et al*., 2008).

2.8 Synthesis of Zeolites from coal fly ash

Recent investigations have shown the potential of fly ash as a raw material for synthesis of various types of zeolites. The conversion of fly ash to zeolite has gained importance due to intensive research on zeolite and growth in geological materials such as volcanic rock and clay minerals. High content of reactive materials like alumino silicate makes it interesting starting material for the synthesis of zeolite with a wide range of applications. Various

methods of synthesis of zeolite from fly ash have, so far, been invented and patented. Some of the important techniques are alkali fusion followed by hydrothermal treatment, slurry method and molten salt method (Grutzeck and Siemer, 1997). Fusion method is found to be the most efficient and a general method for synthesis of zeolite NaX-type, beta-type, and A-type from a large variety of fly ash.

A modified fusion process to synthesize zeolite NaX from fly ash was studied by Chang *et al*. (2000). It was found that the addition of aluminium hydroxide to the fused fly ash solution followed by hydrothermal treatment at 60^0C produced single–phase zeolite A and NaX depending on the source of the received fly ash. The result confirms that the quantity of dissolved aluminium species is critical for the type of zeolite formed from fused fly ashes.

Sutarno *et al*., (2007) synthesized faujasite or zeolite NaX from fly ash and its application for hydrocracking catalyst of heavy petroleum distillates has been studied. Zeolite NaX was synthesized from fly ash by hydrothermal reaction in alkaline solution via combination of reflux treatment of fly ash with HCl and fusion with NaOH. Ojha *et al*., (2004) synthesized NaX-type zeolite by alkali fusion followed by hydrothermal treatment. Querol *et al*., (2002) synthesized zeolitic material from fly ash using two different methodologies: (a) impure zeolitic material obtained by direct conversion from different fly ashes, and (b) a high purity 4A-X zeolite blend synthesized from the silica extracts obtained from the Meirama fly ash.

Fukui *et al*., (2003) studied the effects of NaOH concentration on the crystal structure and the reaction rate of zeolite synthesized from fly ash with a hydrothermal treatment.

Zeolites were synthesized by Rungsuk *et al*., (2006) through fusion method. The synthesis conditions were optimized to obtain the product with high cation exchange capacity (CEC). CFA was mixed with NaOH at various ratios and the results revealed

that the optimal ratio between CFA and NaOH. Muniz *et al*., (2010) studied solid residues resulting from the active treatment of acid mine drainage with coal fly ash were successfully converted to zeolite-P under mild hydrothermal treatment conditions. Scanning electron microscopy showed that the zeolite-P product was highly crystalline. The product had a high cation exchange capacity (178.7 meq/100 g) and surface area (69.1m^2/g) and has potential application in wastewater treatment.

Muniz *et al*., (2010) synthesized beta and ZSM-11 zeolitic material by crystallization hydrothermal treatment method using coal fly ash. Tetraethylammonium hydroxide (TEAOH) and Tetrapropylammonium bromide (TPAB) were used as structure directing agents. Musyoka *et al*., (2011) synthesized zeolite A by crystallization hydrothermal treatment method using coal fly ash.

2.9 Factors affecting Adsorption

The possible interactions and their strength between zeolite and proteins are complicated. They could consist of acid-base reactions. The simplest method of protein immobilization is adsorption. Adsorption of proteins on solid surfaces is a very important phenomenon since it is a common application in many fields such as biology, medicine, biotechnology, and food processing and sometimes they play an important role in a system's performance. In particular many chromatographic separations, such as hydrophobic, displacement, and ion-exchange chromatographies, are based on the tendencies of proteins for the support. Protein adsorption at implanted biomaterials is believed to play an important role in determining their biocompatibility with various biological systems. Another importance of protein adsorption onto solid surfaces is the extensive use of immunodiagnostic reagents, in which antibodies are adsorbed on latex or gold particles or on polystyrene plates. The varied functions,

structures and its interactions are needed to be well understood (Magdassi, 1996 and Nakanishi *et al.,* 2001).

The chemical nature of the adsorbent and its properties (charge, hydrophobicity etc.) determine the mode and strength of binding, as well as, in many cases, the conformational changes in adsorbed protein molecules. For the specific interactions the solid surfaces can be easily modified. Usually, different from fluid surfaces, solid surfaces are not chemically or energetically uniform and their heterogeneity may result in non-uniform adsorption of protein layers. Finally, during adsorption from solutions competition between a protein and a solvent takes place. A large number of different carriers for protein adsorption have been described: glass, silicas, clays, insoluble inorganic salts, ceramics, metals and metal oxides, molecular sieves , carbon, many types of neutral and charged polymers, and carriers of mixed types, obtained by 64 deposition of lipid layers (e.g., lecithin and cholesterol) on a solid support (Magdassi,1996).

There are several techniques used for observing protein adsorption behaviour. The conventional technique used to determine the adsorbed amount is measurement of the decrease in solute concentration after treating with adsorbent. Besides, the adsorbed amount and their changes are obtained by ellipsometry due to the change in polarized light state on reflection. Fluorescence spectroscopy and Fourier transform infrared spectroscopy determine the conformation of proteins adsorbed on surfaces with respect to the change in spectrum. With atomic force microscopy, three dimensional image of protein adsorbed surface can be obtained. The amount of irreversibly adsorbed proteins is measured by radioisotope labeled molecules. The amount and thickness of adsorbed proteins are obtainable with radiotracer technique (Nakanishi *et al.,* 2001). The factors influencing the functional properties of proteins and the ability to regulate these properties are of great importance. These properties determine the adsorption behaviour of proteins. The main molecular properties of proteins are

size, charge, structural properties, stability, amphipathicity, and lipophylity which are responsible for their surface activity. Proteins form multiple interaction points with the surface (e.g., 77 contact points for an albumin molecule and 703 contact points for the fibrinogen molecule adsorbed on silica). The irreversibility of protein adsorption is usually seen in case of multipoint binding if irreversible denaturation is not governing in system. The rates of desorption are much lower than those of adsorption, and in many cases it is often impossible to obtain the equilibrium state for desorbing the adsorbed protein. In other words, the formation of one or several bonds with the surface increases the probability of adsorption of closer sites of the identical molecule. As a result, desorption of a protein molecule requires the simultaneous breakage of a large number of bonds. This corresponds to a considerable difference between the activation energies for the adsorption and desorption processes (Magdassi, 1996).

The chemical differences are also very important because the balance of polar, nonpolar and charged amino acid side chains determines the surface activity of proteins i.e., the possibility and mode of their location at interfaces of different types. Another very important property of proteins is the hydrophobicity. It influences adsorption and orientation of proteins at interfaces and in many cases correlates with surface activity (Magdassi, 1996).

2.10 Desorption study

The desorption ability of the alpha amylase adsorbed on the surface of the natural zeolites were investigated. Few studies concerning the desorption of proteins from zeolites and indicating that, polyethylene glycol and ethyl alcohol are good candidate for desorption eluents for proteins (Sakaguchi *et al.,* 2005 and Chiku *et al.,* 2003). However, the analytical method is a critical point when studying with polyethylene glycol (PEG), but PEG does not work well with the reagents used in Lowry method for protein quantification (Miller *et al*.,

2005). The complex nature of protein mixture prevents the sensitive detection of real protein concentration inside the medium. Thus, eluents other than PEG were studied and analyzed. Desorption of proteins from zeolites is a new area attracting the attention in protein engineering studies (Sakaguchi *et al*., 2005 and Chiku *et al.*, 2003). In the present study, eluents such as alcohol 50%, 70% and 100% were investigated so as to desorb alpha amylase from the adsorbed surface of synthesized zeolites.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Reagents and materials in zeolites synthesis

The coal fly ash (CFA) was obtained from GNDTP (Guru Nanak Dev Thermal Power Plant), Bathinda, in sterile polyethene bags and was stored at room temperature until used. Hydrochloric acid, TM-40 colloidal silica, silicon dioxide, sodium hydroxide, sodium aluminate, tetraethylammonium hydroxide (TEAOH), ammonium chloride and deionized water were used for zeolite synthesis. All the chemicals were procured from Sigma, MO, U.S.A and Himedia, Mumbai were of highest analytical grade available.

3.2 Synthesis of zeolite NaX (Fathizadeha *et al.,* 2011)

Prior to any treatment, the unburnt carbon (4–6%) along with other volatile materials present in coal fly ash was removed by calcination at 800°C for 2 hours. Mixture of sodium hydroxide and fly ash (calcined and HCl treated) in a pre-determined ratio, was milled and fused in a stainless steel tray at different temperatures ranging from 500–650°C for 1 hour. The sodium hydroxide to fly ash ratio (by weight) was varied from 1–1.5. The resultant fused mixture was then cooled to room temperature, ground further and added to water (10 g fly ash/100 ml water). The slurry thus obtained, was agitated mechanically in a glass beaker for 8 hours. It was then kept at around 90°C for 6 hours, without any disturbance. The resultant precipitate was then repeatedly washed with distilled water to remove excess sodium hydroxide, filtered and dried at room temperature for 24 hours for further characterization.

3.3 Synthesis of zeolite beta (Muniz *et al.,* 2010)

CFA (9 g) was mixed with 10.8 g of NaOH and the resulting mixture was heated at 600°C for 1 hour. In case of β zeolite synthesis, homogeneous mixtures with molar composition of $6.5Na_2O:6.0(TEA)_2O:30SiO_2:Al_2O_3:840H_2O$ were prepared, by adding colloidal silica (3.4 g) to a mixture comprising of (0.68 g) aqueous solution of TEAOH, (0.73 g) NaOH pellets and

95.2 ml water, this slurry was added in the mixture of coal fly ash and NaOH. After adjusting the pH to 12±0.2 with concentrated HCl, the reaction mixture was subjected to crystallization at 140°C. The time of crystallization was varied from 4-95 hours (four days). The solid product was recovered by filtration, washed thoroughly with deionized (DI) water, and then dried at 100°C for further characterization.

3.4 Synthesis of zeolite A (Musyoka *et al.*, 2011)

Zeolite A was synthesized by fusing fly ash with sodium hydroxide followed by a hydrothermal reaction. A homogenous mixture of fly ash and sodium hydroxide was prepared by mixing fly ash and sodium hydroxide in a ratio of 1:1.2. The resultant mixture was fused at 550°C for 1.5 hours. Thereafter, the fused fly ash was cooled and thoroughly mixed. During the synthesis, 10 g of the fused fly ash was mixed with 50 ml of ultra pure water and stirred for two hours (Mixture A). A solution of sodium aluminate was prepared by mixing sodium aluminate powder with sodium hydroxide in a ratio of 1:2 and stirred using a magnetic stirrer for 30 minutes (Mixture B). 20 ml of mixture B was added to the mixture A and stirred for another ten minutes. The resulting slurry was then subjected to crystallization at 100°C for 20 hours. After the hydrothermal crystallization, the solid crystalline product was recovered by filtration and was washed thoroughly till the filtrate reached pH 9—10 and dried at a temperature of 100°C for 12 hours.

3.5 Characterization of zeolite

3.5.1 X-ray Diffraction

XRD of all the three synthesized zeolites and their commercial counterparts was obtained from SAIF PU, Chandigarh; the instrument used was the (Philips Electronic Instruments X-ray generator Netherlands). Zeolite materials being crystalline solids have characteristic

diffraction patterns that can be used to identify their exact structure and to determine their degree of crystallinity. Operating conditions involved the use of CoK*a* radiation at 45 kV and 40 mA. The samples were scanned from 5–60° (2*q*, where *q* is the angle of diffraction). Quantitative measure of the crystallinity of the synthesized zeolite was made by using the summed heights of major peaks in the X-ray diffraction pattern (Szostak 1976). The major peaks were selected specifically because they are least affected by the degree of hydration of samples and also by others. The percentage crystallinity was measured using the formulae:

% Crystallinity = (sum of the peak heights of unknown material) × 100/ (sum of peak heights of standard material).

The crystal size is calculated according to scherer's equation:

$$r = k \lambda / \beta \cos\Theta$$

Where K is the shape factor, λ is the x-ray wavelength, typically 1.54 A^0, β is the line broadening at half the maximum intensity (FWHM) in radians, and Θ is the Bragg angle.

3.5.2 SEM (Scanning Electron Microscope)

The crystal morphology of fly ash and zeolite NaX was examined with a scanning electron microscope (LEO 435 VP, Netherlands) with resolution 4.0nm, magnification of 10 X to 300,000 X and having a SE detector. The samples were coated with gold using a polaron sputter coater (15 A Quorum technologies, U.K).

3.6 Production of Alpha amylase from *L.lactis* (Wasko *et al.,* 2010)

3.6.1 Bacterial strain and culture conditions

Overnight grown culture of *L.lactis* (1%) was inoculated in 100 ml of MRS (Man Ragosa sharpe) medium with 1% of soluble potato starch (Himedia, Mumbai) as sole carbon source. The fermentation process was carried out in stirred tank fermentor (Fermac 360, Electrolab, INDIA) with a capacity of 3 litres. MRS media (2.5 litres) was inoculated with 1% of inoculum and the growth conditions were maintained at 30^0C, 150 rpm at constant pH of 6 for 72 hours. Samples were withdrawn at regular intervals throughout the fermentation process and the supernatant was analyzed for enzyme activity.

3.6.2 Enzyme activity assay

Enzyme activity was measured by amylase starch assay (Gupta *et al*., 2010). Culture broth was centrifuged at 8000 rpm for 10 minutes. Further, the 0.1 ml of supernatant was added to the 0.8 ml of substrate solution (1.2 % starch in 100ml of citrate phosphate buffer of pH 5.5). Mixture was heated for 30 minutes at 55^0C for the complete degradation reaction between enzyme and starch. Then 0.1 ml H_2SO_4 was added to the above mixture in order to stop the reaction. 2.4 ml of iodine solution was added to 0.1 ml of above solution and absorbance was recorded at 620 nm. One unit of amylase activity was defined as the amount of enzyme that released 1 μmol of reducing end groups.

The standard curve of commercially available alpha amylase was plotted for comparison with alpha amylase produced from bacteria (Annexure, Fig.A)

3.7 Optimization studies

Adsorbent dosage, contact time and enzyme concentration were the important parameters to find out the optimum conditions for the adsorption as well as purification of alpha enzyme by zeolites.

3.7.1 Dose optimization

To study the effect of three synthesized zeolites dose, experiments were run using six different concentrations of zeolites (NaX, Beta and A) i.e 0.05 mg, 0.1 mg, 0.25 mg, 1 mg, 10 mg and 20 mg. Zeolite was added to the supernatant containing alpha amylase, vortexed for 30 sec and was shaken manually for maximum time to facilitate adsorption. Then the mixture was centrifuged at 8,000 rpm for 5 min. Supernatant was collected and residual amylase was measured by amylase starch assay (method described earlier). To analyze the efficiency of the zeolite dose, the reduction in the enzyme activity was estimated. Each experiment was performed in triplicate.

3.7.2 Optimization of contact time

The contact time range was selected as 0-30 minutes. Optimum dose of each modified adsorbent was added to the culture supernatant. Each reaction mixture was vortexed for 30 seconds and then simply shaken for respective time. Then the mixture was centrifuged at 8,000 rpm for 5 min at 4^0C. Supernatant was collected and residual amylase was measured by amylase starch assay. Each experiment was performed in triplicates

3.7.3 Optimization of enzyme concentration

Effect of enzyme concentration was studied by varying the concentration of enzyme from 20 U to 60 U. Optimum dose of each modified adsorbent was added to culture supernatant. Each reaction mixture was vortexed for 30 seconds and then simply shaken for respective time. Then the mixture was centrifuged at 8,000 rpm for 5 min at 4^0C. Supernatant

was collected and residual amylase was measured by amylase starch assay. Each experiment was performed in triplicates.

Percentage reduction in enzyme activity with varying the adsorbent dose, time and enzyme concentration was calculated using:

$$\text{Percentage reduction} = C_0 - C_1 / C_0 \times 100$$

C_0 =Initial enzyme activity

C_1 = Final enzyme activity

3.8 Recovery of α- amylases

The optimum zeolite dose (1 mg/ml) was used to treat the culture broth for optimum time (10 min) with optimum enzyme concentration. Further, the culture broth was centrifuged for 8000 rpm for 5 minutes. The obtained cell pellet which contained α-amylase bounded to zeolites was further processed for separation of purified α-amylase. 50%, 70% and 100% ethanol was added to the pellet for precipitation, then the mixture was centrifuged and enzyme activity in the supernatant was measured by alpha amylase assay. Further, the stability effect of temp, pH and agitation was checked on the activity of purified enzyme.

3.9 Stability studies of purified enzyme (Petrov *et al.,* 2008)

The stability of purified amylase was determined at various pH values (ranging from 2.0 to 9.0) in 0.1M acetate buffer. To determine thermo stabilities of the enzyme, the samples were pre incubated in 0.1M acetate buffer at the optimum pH and temperatures ranging from 20 to 50°C for 1 hour. The samples were then chilled on ice for at least 30 min, and residual activity was determined at pH 4.5 and the temperature of 35^0C and agitation of 8000 rpm using the amylase starch assay (method described earlier)

3.10 Fourier transform infrared spectroscopy (FTIR)

FTIR is most useful technique for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. 1 mg of freeze-dried partially purified zeolite NaX and alpha enzyme adsorbed on zeolite NaX was grounded with 100 mg of KBr and pressed with 7500 kg for 30 seconds to obtain translucent pellets. Infrared absorption spectra were recorded on a FTIR system (Perkin Elmer - Spectrum RX-IFTIR, U.S.A) with a spectral resolution and wave number accuracy of 4 and 0.01cm^{-1}, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSIONS

In the present study, zeolites were synthesized and applied to evaluate their efficiency for purification of alpha amylases. Since, alpha amylases have profound importance in various industrial applications, an innovative purification is desirable.

4.1 Synthesis and characterization of zeolites

Increase in temperature and agitation promotes the synthesis of zeolites with small crystals of µm range. It is well known that synthesis time directly affect the crystallization process. In the present study, the NaX zeolite was obtained after 6 hours of hydrothermal treatment, while zeolites beta and A were obtained after 95 and 20 hours of crystallization, respectively. Higher product yield and large particle size was obtained at longer synthesis time. Factors such as temperature, agitation, fusion time and alkali treatment were taken into consideration during zeolite synthesis. Yield obtained of zeolites NaX, beta and A were 58 g, 15g and 57 g, respectively (Muniz *et al*., 2010).

The coal fly ash contains mainly SiO_2, Al_2O_3 and some amount of Fe_2O_3 and the oxides of Mg, Ca, P, Ti etc (Fukui *et al*., 2003). Physico-chemical composition of fly ash samples was obtained from EDAX depicted in Table 1.

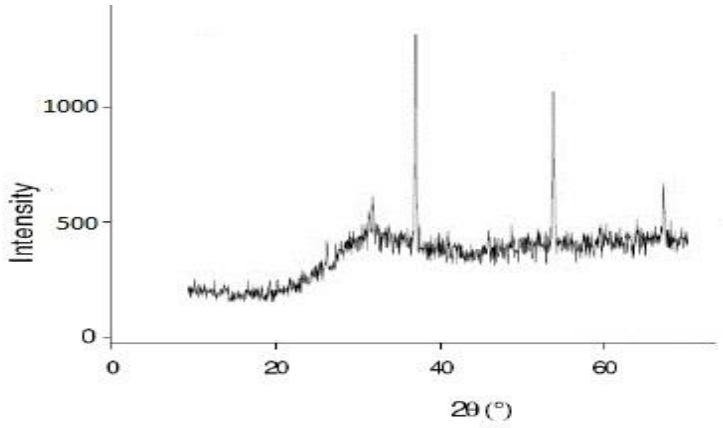
Table 1 Physico-chemical composition of coal fly ash

Components	Composition
Na_2O	2.12
Al_2O_3	30.01
SIO_2	55.19
K_2O	1.40
CaO	0.77
TiO_2	2.74
Fe_2O_3	4.58
BaO	1.28
MgO	1.91

4.2 X –ray Diffraction

The XRD pattern suggested that the samples prepared from coal fly ash were more crystalline than the precursor material. In the case of Zeolite NaX (Fig.1), it was found that newly synthesized zeolite was crystalline in nature but lesser than its commercially available counterpart. Fig.2 and 3 depicted that XRD patterns of zeolite beta and zeolite A were found to be similar to their commercially available counterparts. Results revealed sharp and intense peaks in the X-ray diffraction analysis (Querol *et al*., 2002).

(a)



(b)

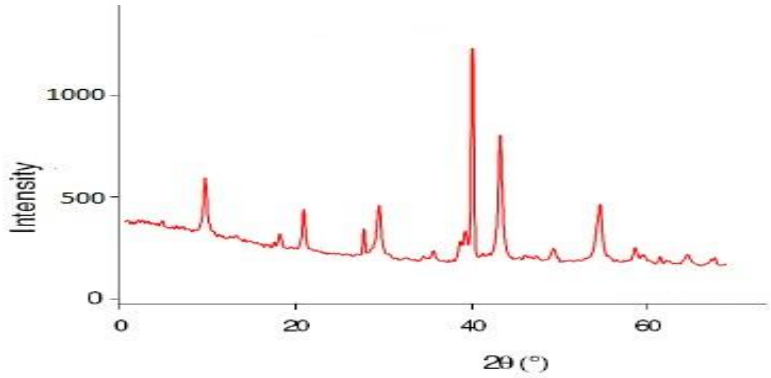


Fig.1 XRD patterns of commercial 13X zeolite (a) and synthesized zeolite NaX (b).

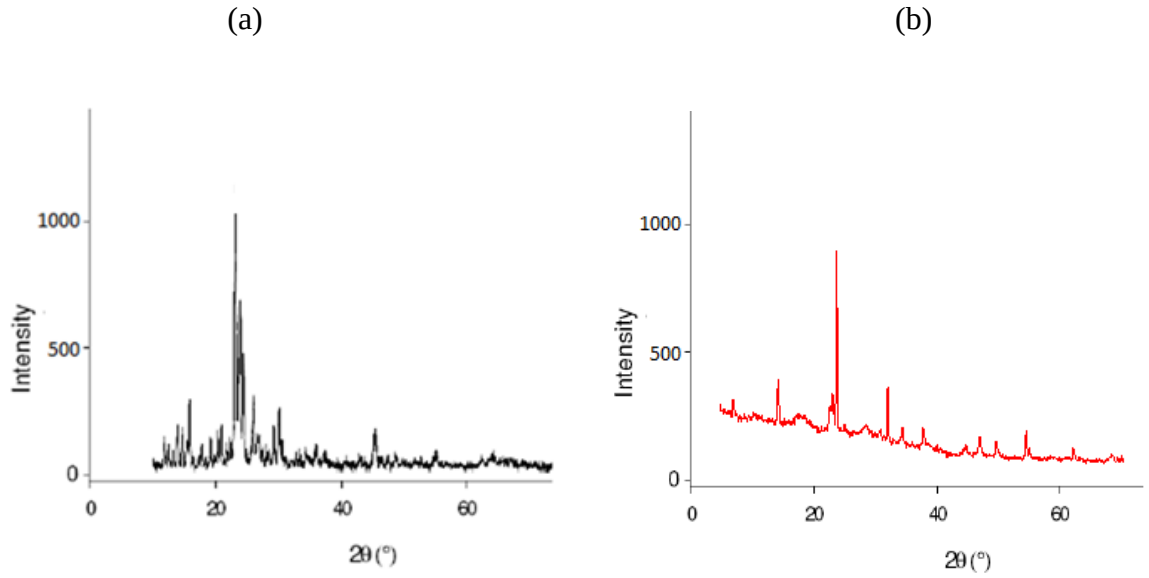


Fig. 2 XRD patterns of commercial zeolite beta (a) and synthesized zeolite beta (b).

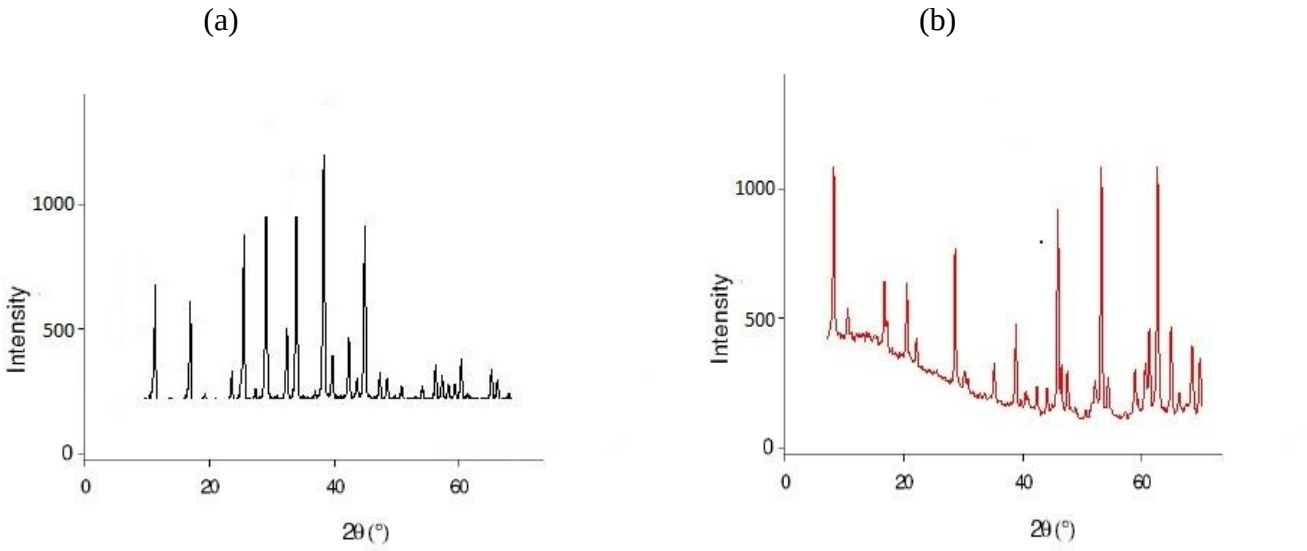


Fig. 3 XRD patterns of commercial zeolite A (a) and synthesized zeolite A (b).

The diffraction of X-rays from zeolite crystallites produce a scattering pattern which is specific of periodic arrangements of regular arrays of atoms or ions located within

the zeolite structure. XRD is important for inferring crystal size, crystalline phase purity and measurement of solid sample amorphous or crystalline structure (Kim *et al.*, 2001).

Table 2 Peak list and particle size analysis of (a) zeolite NaX (b) zeolite A (c) zeolite beta.

(a)

Peak	Θ(pos)	B(FWHM)	Particle size(µm)
1	10.1989	0.2007	7.7
2	38.0242	0.2342	6.9
3	28.1226	0.1840	8.8
4	14.0650	0.4015	3.8

(b)

Peak	Θ(pos)	B(FWHM)	Particle size(µm)
1	6.1882	0.1004	15.3
2	16.224	0.1338	11.66
3	24.767	0.2007	7.85
4	26.265	0.1171	13.5

(c)

Peak	Θ(pos)	B(FWHM)	Particle size(µm)
1	12.151	0.1506	10.2
2	29.666	0.3346	4.7
3	35.315	0.2007	8.0
4	50.204	0.1338	12.3

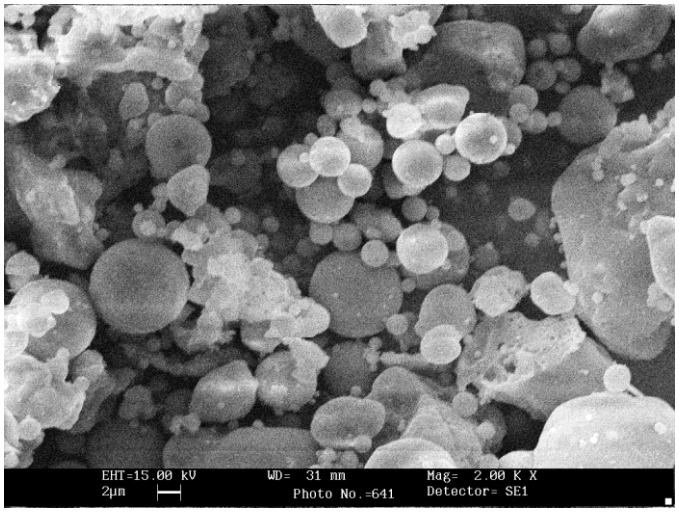
The particle size was calculated by scherrer's equation for zeolites NaX, A and beta. The average particle size for zeolite NaX, A and beta was 6.8 µm, 12.065 µm and 8.8 µm, respectively. Results were in accordance to the previous studies where the particle size of newly synthesized zeolites was found to be similar to that of commercially available zeolites (Grutzeck and Siemer, 1997, Musyoka *et al*., 2011).

The reference sample was assumed to be 100% crystalline. It was found that zeolite NaX, beta and A was 81.2 %, 70.5 % and 75 % crystalline, respectively.

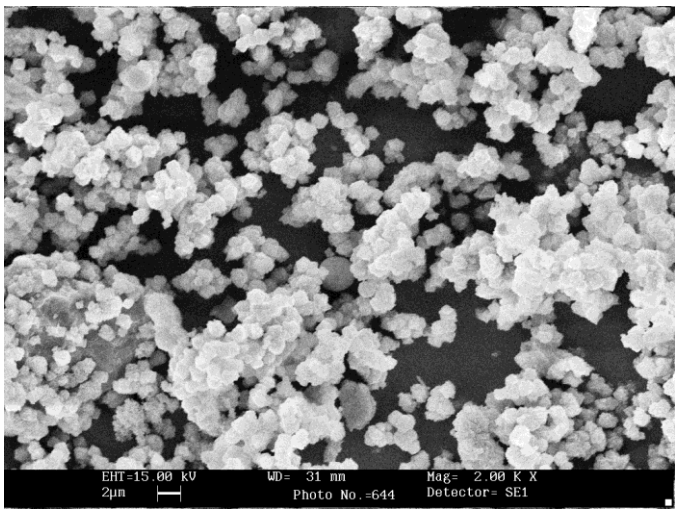
4.2 Scanning Electron Microscope analysis (SEM)

The scanning electron micrographs (SEM) of the original fly ash, treated fly ash (synthesized zeolite) and commercial zeolites are shown in Fig. 4(a), (b) and (c), respectively. The absence of the spherical particles in treated fly ash (as evident from the SEM) indicated high conversion of fly ash to crystalline zeolite on hydrothermal treatment. The crystal structure of the synthesized zeolite was determined to be distorted octahedral (Charnell 1971, Bogomolov and Petranovsky 1986).

(a)



(b)



(c)

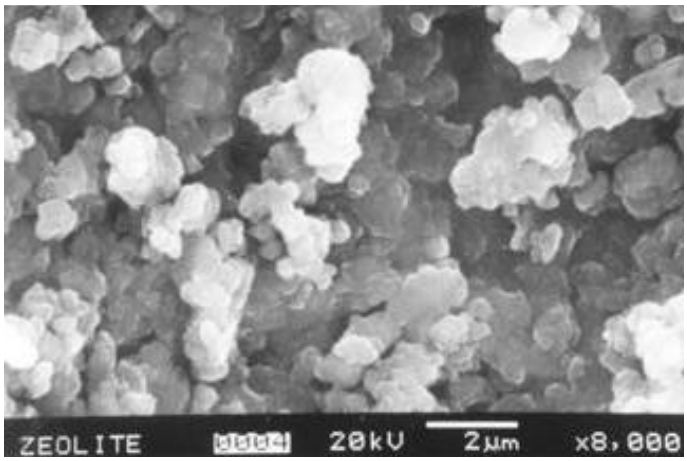


Fig.4 Scanning electron micrographs of (**a**) fly ash, (**b**) synthesized zeolite NaX and (**c**) commercial 13X zeolite.

4.3 Production of Alpha amylase

A lactic acid bacterium has been considered to be the most important sources of alpha amylases and had been used for enzyme production (Wasko *et al*., 2010).

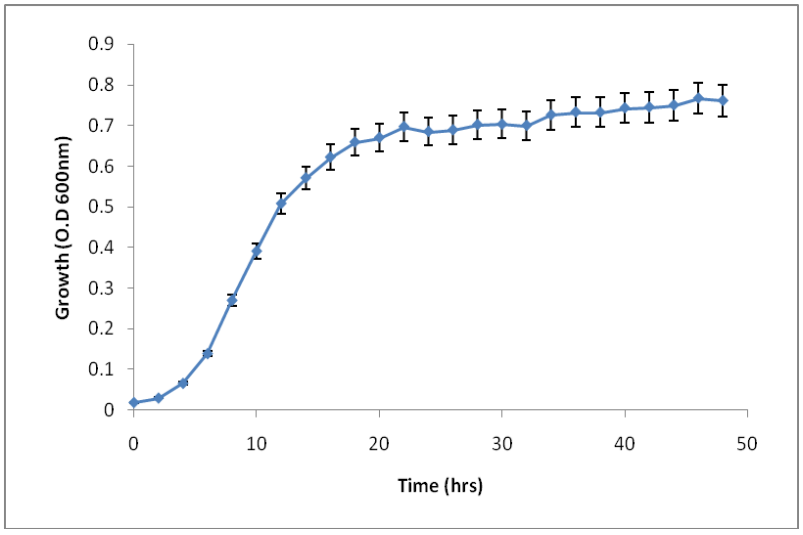


Fig. 5 Growth kinetics of *L. Lactis.* (Error bar represents the standard deviation from mean value of triplicate experiments).

Fig. 5 showed the growth kinetics of *L. lactis* and results revealed that amylase production by the bacteria was found to be growth-associated as the maximum enzyme production (3.2 U/ml) was observed at 48 hours.

4.4 Adsorption dynamics of synthesised zeolite crystals

4.4 .1 Optimization of dose

The synthesized zeolites NaX, beta and A were used as adsorbents. Optimization of their dosage was one of the most important parameter in determining the optimum condition for the adsorption process. The series of experiments were run to determine the optimum adsorbent dosage. The study was carried out by taking the adsorbent dose in range of 0.05 mg/ml-20 mg/ml for all the zeolites NaX, beta and A. The time range was considered in the dose optimization experiment was 1-20 minutes.

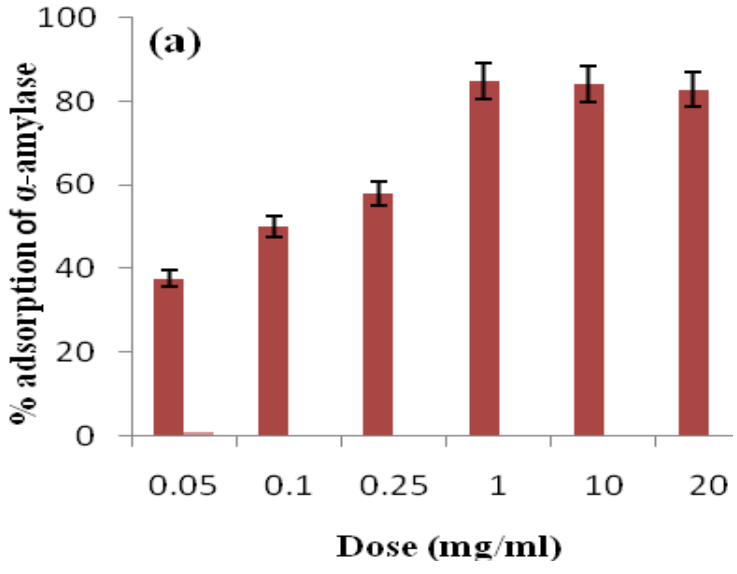


Fig. 6(a) Optimization of adsorbent (zeolite NaX) dose for maximum adsorption of alpha amylase. (Error bar represents the standard deviation from mean value of triplicate experiments).

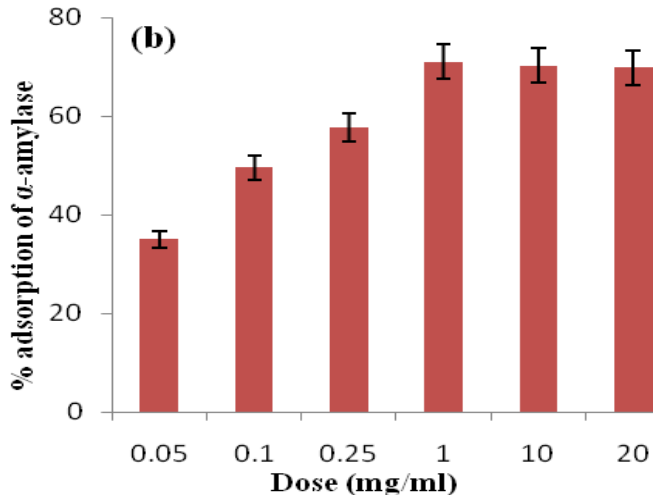


Fig. 6(b) Optimization of adsorbent (zeolite beta) dose for maximum adsorption of alpha amylase.(Error bar represents the standard deviation from mean value of triplicate experiments).

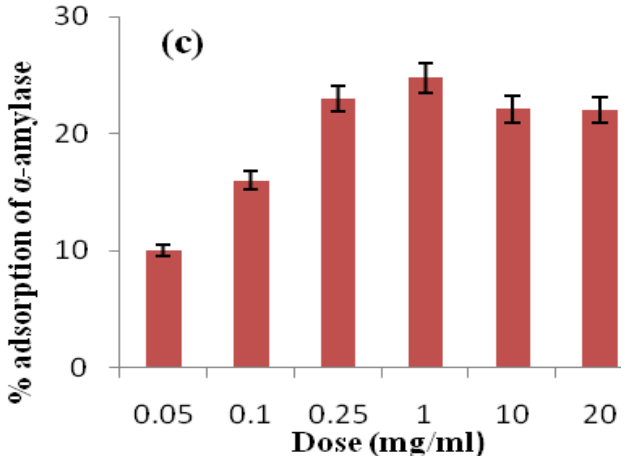


Fig. 6(c) Optimization of adsorbent (zeolite A) dose for maximum adsorption of alpha amylase. (Error bar represents the standard deviation from mean value of triplicate experiments).

Results indicated that at adsorbent dosage of 0.05 to 20 mg/ml, there was an increase in adsorption when amylases were treated with a dosage of 1 mg/ml. The binding of residual enzyme with zeolites initially increased with higher doses of the zeolites. At dose of 20 mg/ml of zeolites, binding of residual enzyme with zeolites was observed to decrease. Overall zeolite NaX exhibited maximum extraction of residual enzyme as compared to zeolite beta and zeolite A, shown in Table 3.

Table 3 Alpha amylase adsorption on zeolites NaX, beta and A

Dose (mg/ml)	% adsorption by Zeolite A	% adsorption by Zeolite Beta	% adsorption by Zeolite NaX
0.05	10.0	35.0	37.7
0.1	16.0	49.6	50.2
0.25	23.0	57.7	57.9
1	24.7	71.1	84.8
10	22.1	70.2	84.1
20	22.0	69.8	82.7

From the above study it was concluded that with increasing adsorbent dosage, more active sites were available, making amylases penetration to the adsorption sites easier and increased the adsorption capacity. The adsorption of alpha amylases remained almost constant at adsorbent dosage higher than 1 mg/ml, thus this concentration was selected as optimum for adsorption as no significant change occured. This might be due to the saturation i.e all the active sites were occupied by the amylases and no further adsorption can occur. Therefore, the optimum dose of adsorbents used for further studies was 1 mg/ml.

4.4.2 Optimization of contact time

Adsorption of alpha amylase was studied over various time intervals. Besides the effect of adsorbent dosage, the contact time also plays a crucial role in adsorption process for alpha amylase from bacterial culture. As the adsorption process requires proper contact

between the adsorbent and adsorbate, vortexing (30 sec) was done followed by shaking to increase the effectiveness of the process. It was observed that optimum adsorption of alpha amylase on all the three zeolites was achieved with in the first 10-20 minutes and remained fairly stable thereafter. The maximum binding of alpha amylase with time was observed with crystalline zeolites NaX, Beta and A and it was analyzed that zeolite NaX was more efficient in extraction of alpha amylase when compared to both zeolite beta and A. The maximum recovery percentage was achieved by zeolite NaX (84%) while percentage for recovery with zeolite Beta was 74% and with zeolite A was 39%.

It was observed that with increase in time of contact, the adsorption of the alpha amylase increased and the highest level of adsorption was observed at 15-20 minutes.

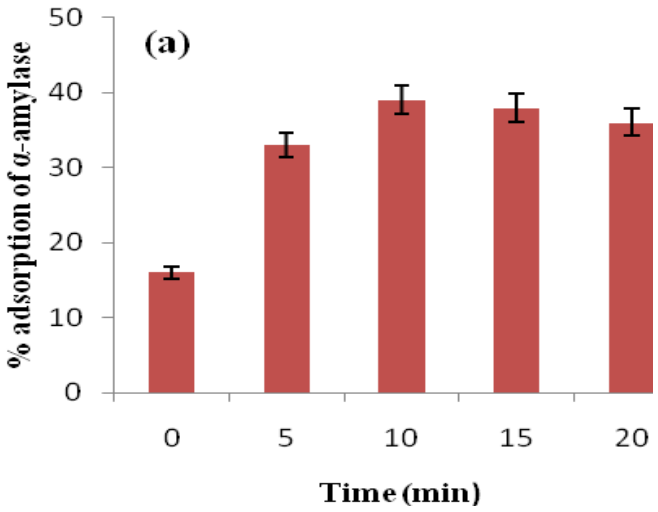


Fig. 7(a) Optimization of time for maximum adsorption of alpha amylase with zeolite A. (Error bar represents the standard deviation from mean value of triplicate experiments).

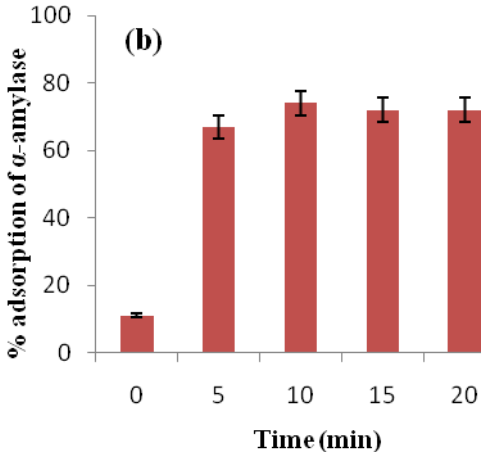


Fig. 7(b) Optimization of time for maximum extraction of alpha amylase with zeolite beta. (Error bar represents the standard deviation from mean value of triplicate experiments).

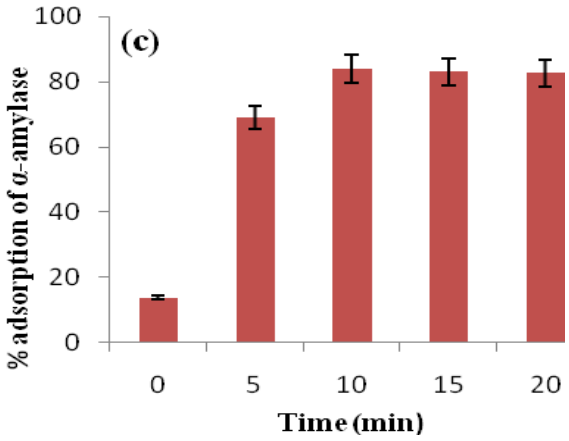


Fig. 7(c) Optimization of time for maximum extraction of alpha amylase with zeolite NaX. (Error bar represents the standard deviation from mean value of triplicate experiments).

Results of time optimization experiments for zeolite NaX and beta are shown in Fig. 7 (b and c). The same pattern as observed in the case of zeolite A, was obtained and results showed that maximum binding of alpha amylase with zeolite NaX and beta occured after 10 minutes and afterwards it remained stable.

4.4.3 Optimization of enzyme concentration

Adsorption of alpha amylase was studied under the effect of increasing enzyme concentration. Results indicated that optimum alpha amylase adsorption was achieved at enzyme concentration of 50 U–60 U and remained stable thereafter at 1 mg/ml of adsorbent dose and 15 minutes of contact time.

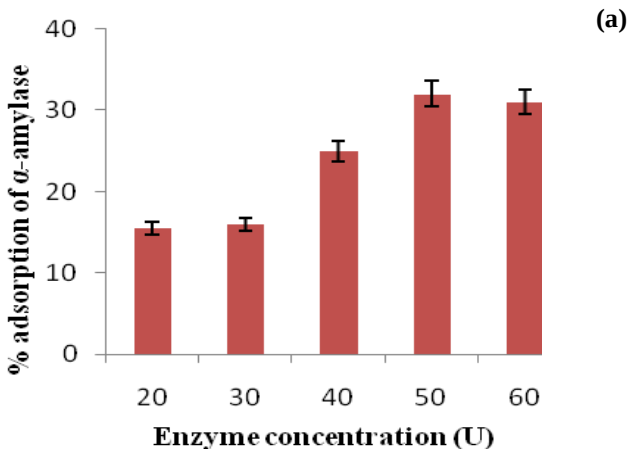


Fig.8 (a) Optimization of enzyme concentration for maximum extraction of alpha amylase with zeolite A. (Error bar represents the standard deviation from mean value of triplicate experiments).

Fig. 8 (a) depicts that maximum extraction of alpha amylase with zeolite A occured at enzyme concentration of 50 U and it remained fairly stable thereafter.

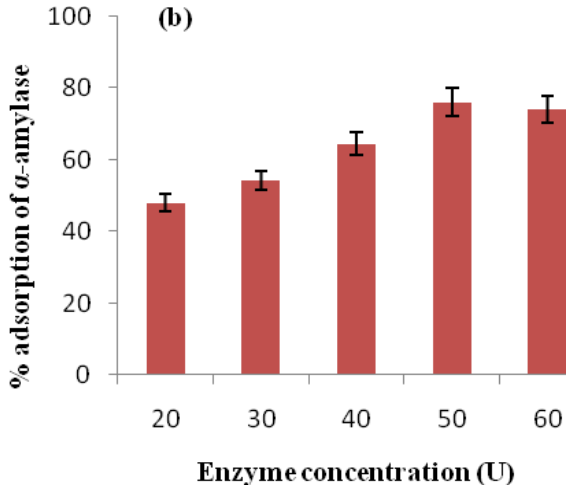


Fig. 8(b) Optimization of enzyme concentration for maximum extraction of alpha amylase with zeolite NaX. (Error bar represents the standard deviation from mean value of triplicate experiments).

Fig. 8(b) showed that maximum extraction of alpha amylase with zeolite NaX occured at enzyme concentration of 48 U, no change was noticed thereafter.

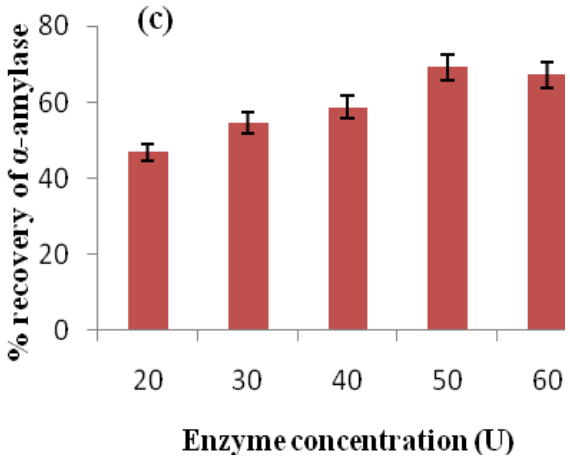


Fig. 8 (c) Optimization of enzyme concentration for maximum extraction of alpha amylase with zeolite beta. (Error bar represents the standard deviation from mean value of triplicate experiments).

Fig. 8 (c) showed that maximum extraction of alpha amylase with zeolite beta, which occured at enzyme concentration of 47 U and following which it remained fairly stable.

Protein adsorption at the solid/liquid interface is influenced by the surface charge of the molecule and the surface charge of the solid. The overlap of the electrical double layers at the sorbent and the protein surfaces results in electrostatic attraction if they have opposite charges and in repulsion if their charges have the same sign (Akgul *et al*., 2008). Besides, of great importance is the repulsion between the protein molecules in adsorbed layers, which is minimized at their isoelectric points.

The charge, its density and 65 distributions in the protein molecule have a great affect on the surface activity. Proteins frequently show greater surface activity near the isoelectric point (pI), because coulombic repulsion between the same charged adsorbed molecules decrease. In case of adsorption at ionic surfaces the main factor is the net opposite charge of the protein molecule with the adsorbed surface, which may contribute to the enthalpic part of the adsorption free energy. Also, heterogenous distribution of ionic sites on the surface of a protein can lead to attractive electrostatic interactions between the sites and the surface even when the net charge of the protein is same with the surface.

The pH of the solution determines the charge of the proteins that are comprised of both charged amino and carboxyl groups and the interacting surfaces consequently is responsible for the attractive and repulsive effects (Tavolaro *et al.*, 2007, Magdassi, 1996; Krohn and Tsapatsis, 2005, Klint and Eriksson, 1997 and Munsch *et al.*, 2001). Although electrostatic forces are very important, they do not dominate the adsorption process solely. In many cases qm is at a maximum value near the isoelectric points of the protein/sorbent complex. One possible explanation of this behavior is that increased lateral electrostatic repulsion of the equally charged protein molecules prevents the formation of close-packed

monolayers. Another explanation tells that the structural stability of the protein molecules is maximal and the conformational rearrangements are minimal when adsorption proceeds at a pH equal to the pI of the protein/sorbent complex. The dehydration of surface and protein causes release of water molecules which enhances the adsorption of protein on hydrophobic surface. On the contrary, affinity of adsorption onto hydrophilic surface decreases (Magdassi, 1996, and Giacomelli *et al.*, 1999)

In the present work the possible mechanism of adsorption may be concluded through three steps. In the first step, alpha amylase was transferred from the liquid phase to the external surface of the adsorbent material. In the second step, the α-amylase diffused from the relatively small area of the external surface into the macropores, transitional pores and micropores within each adsorbent. Most adsorption occured in the micropores as the majority of surface area is available (Akgul *et al*., 2008). In the third step, the α-amylase adsorbs to the surface in the pore and the actual physical bonding between the adsorbate and the adsorbent surface occurred.

4.5 Characterization of Zeolite NaX after adsorption of alpha amylase

Infrared (IR) spectroscopy can yield information concerning structural details of the material. In general, the IR spectrum can be split into two groups of vibrations: (i) internal vibrations of framework TO_4 units, which are insensitive to the structural vibrations; and (ii) vibrations related to the external linkage of the TO_4 units in the structures. The latter is sensitive to structural vibrations. In general, each zeolite has a characteristic infrared pattern. However, some common features were observed, which include the asymmetric and symmetric stretch, double ring vibrations, T–O bending modes, and possibly pore opening modes. The frequency regions where different kinds of vibrations were located in zeolites are summarized in Fig. 9.

(a)

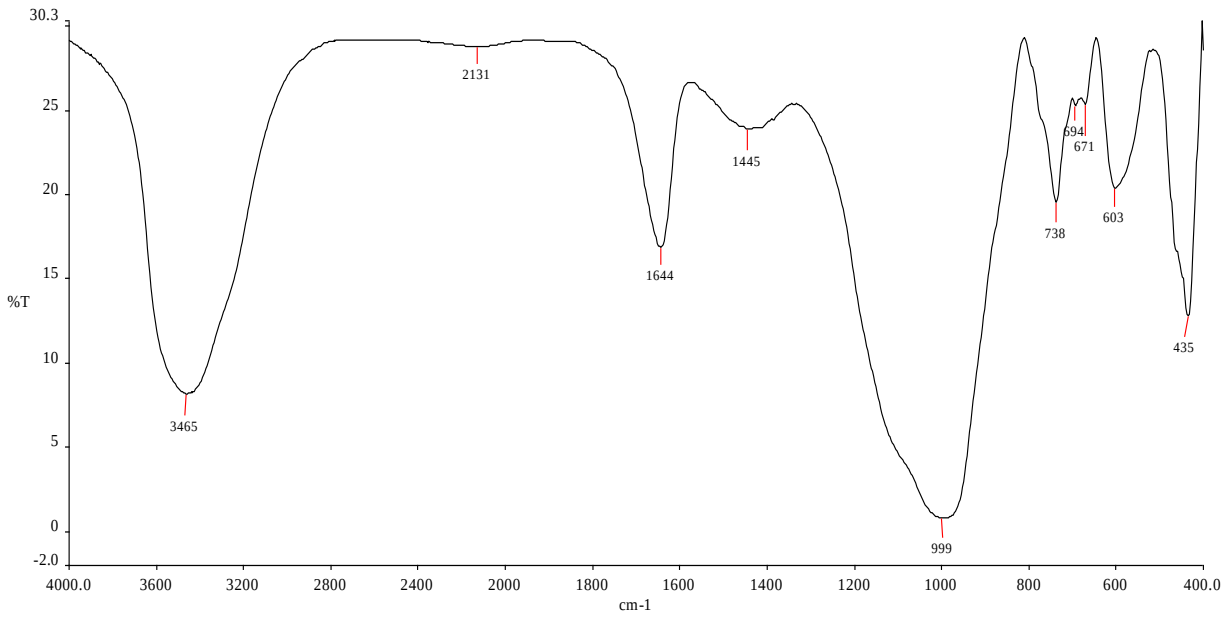


Fig 9. (a) IR spectra of synthesized zeolite NaX.

(b)

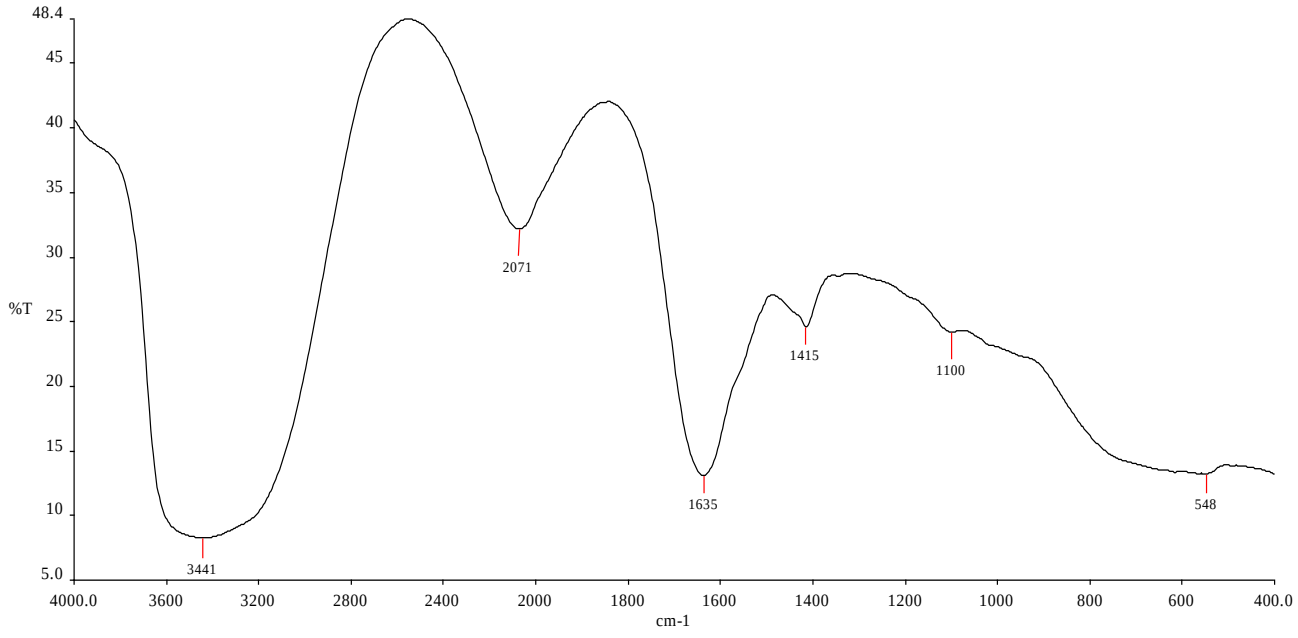


Fig 9. (b) IR spectra of adsorbed alpha enzyme on zeolite NaX.

Difference in the FTIR spectra of zeolite NaX compared to that of enzyme adsorbed on the surface of zeolite NaX revealed the difference in band frequencies. Additional broad and weak bands due to amylase were observed around 1100cm^{-1} and 548 cm^{-1} in case of enzyme adsorbed on zeolite surface (Fig. 9 (b)). The bands around 3465 cm^{-1} and 1644 cm^{-1} in case of zeolite were observed to shift at lower frequencies around 3441cm^{-1} and 1635 cm^{-1} in case of adsorbed enzyme which resulted due to some interaction between zeolite and the enzyme.

4.6 Alpha amylase desorption from zeolite

Desorption ability of alpha amylase from all the three synthesized zeolites was investigated. The eluent used in the present study was alcohol, which has been proved to be a promising eluent by various authors (Miller *et al*., 2005.). Out of the three different percentages of alcohol used, it was found that 100% alcohol led to highest recovery of the enzyme (Fig. 10). Water was also used as another eluent in the study, but the results showed that it was not a good for desorption step. Results revealed that 73% and 64% of alpha amylase was recovered from zeolites NaX and beta respectively after 30 minutes, and thereafter it remained constant. In the case of zeolite A, no significant desorption was observed. As the desorption curve remained constant after 30 minutes to 60 minutes, the study mainly focused on 30 minutes time period. The pH of the buffer solution is important for the desorption efficiency. As the pH values were higher better results were obtained.

Considering, all the desorption studies, it can be concluded that highest amount of purified alpha amylase was recovered from the sample treated with zeolite NaX.

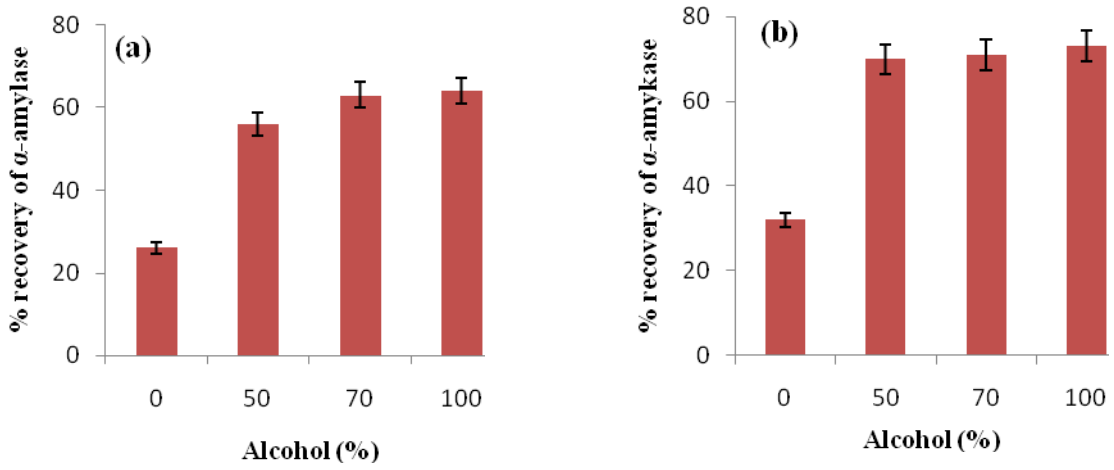


Fig. 10 Alpha amylase recovery from (a) zeolite beta and (b) zeolite NaX. (Error bar represents the standard deviation from mean value of triplicate experiments).

The rates of desorption were much lower than those of adsorption, and in many cases it is often impossible to obtain the equilibrium state for desorbing the adsorbed protein. In other words, the formation of one or several bonds with the surface increases the probability of adsorption of closer sites of the identical molecule (Sakaguchi *et al.*, 2005 and Chiku *et al.*, 2003). As a result, desorption of a protein molecule requires the simultaneous breakage of a large number of bonds. This corresponds to a considerable difference between the activation energies for the adsorption and desorption process (Magdassi, 1996).

4.7 Effect of pH and temperature on stability of purified enzyme

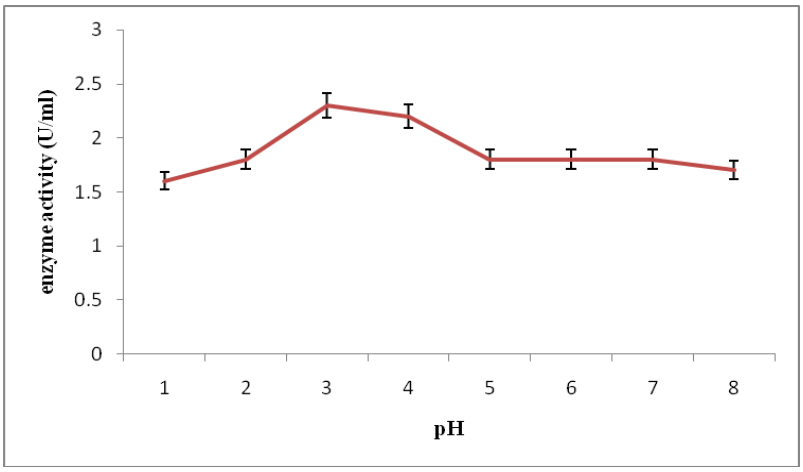


Fig.11 Effect of pH on enzyme activity. (Error bar represents the standard deviation from mean value of triplicate experiments).

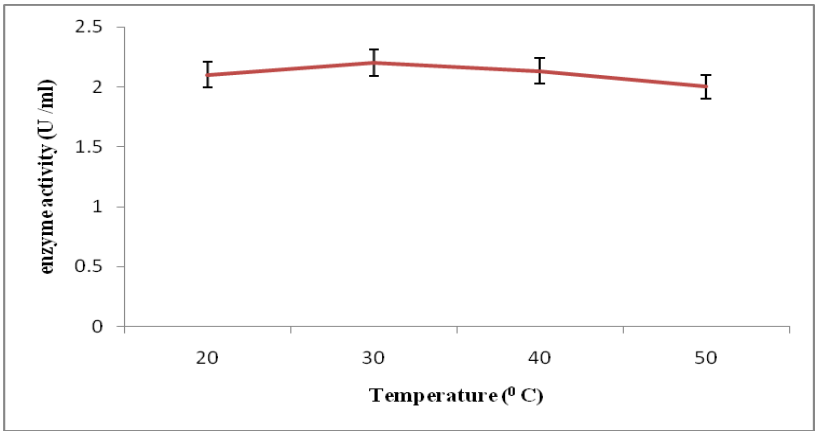


Fig. 12 Effect of temperature on enzyme activity. (Error bar represents the standard deviation from mean value of triplicate experiments).

Results of present study (Fig. 11 and 12) revealed that purified enzyme showed an optimum activity at a lower pH (3.5) and lower temperature (35^0C). Amylases reported by other authors exhibited an optimum activity at pH 5.0-6.4 and at a temperature range from 40^0C to 60^0C. Enzyme was stable at pH 3.5 and temp 35^0C for 60 min, after which period the activity of the enzyme was lost (Gupta *et al.*, 2003)

4.7.1 Effect of agitation

Enzyme production increased as the agitation intensity was increased and found to be maximal at 8000 rpm. Change in the rate of agitation resulted reduction in enzyme stability (Fig. 13).

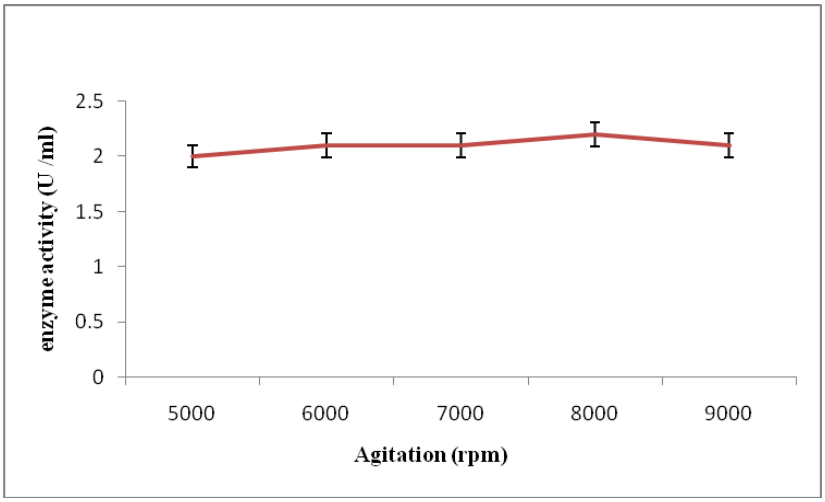


Fig. 13 Effect of agitation on enzyme activity. (Error bar represents the standard deviation from mean value of triplicate experiments).

Probably higher stirring speed above 8000 rpm resulted in mechanical and oxidative stress, excessive foaming, disruption and physiological disturbance of cells, while lower stirring speed seemed to limit oxygen levels along with the lacking of homogeneous suspension of the fermentation.

CONCLUSION

CONCLUSIONS

✓ The three zeolites NaX, beta and A were synthesized from coal fly ash and characterized by XRD, SEM and FTIR techniques, which confirmed their crystalline structure and particle size in µm range.

✓ Approximately 3.2 (U/ml) of alpha amylase was produced by bacterial strain *L.lactis* grown in MRS broth at 30^0 C at 48 hours.

✓ Synthesized zeolites were used to study adsorption of alpha amylase, using various parameters such as dosage of the adsorbent (zeolite NaX, Beta and A), time of contact between adsorbent and adsorbate and effect of enzyme concentration.

✓ Alpha amylase was recovered or purified from zeolites in which alpha amylase was adsorbed during adsorption process. Alcohol proved to be an effective as eluent for the recovery of the alpha-amylases. Among all the three newly synthesized zeolites, NaX showed maximum adsorption and also resulted in maximum recovery of alpha amylase. Further the stability of purified enzyme was checked by several physiochemical parameters such as pH, temperature and agitation.

✓ The described results indicate that zeolite NaX have interesting properties concerning the purification of alpha amylases directly from culture broth. The purification and adsorption capacities of zeolites Beta and A were lower in comparison to zeolite NaX, as the pore size of zeolite compared to the size of amylase molecules favoured the adsorption of alpha amylase where as zeolite beta having larger pores are not effective for adsorption and zeolite A having smaller pores does not favours adsorption in the pores.

✓ Overall results confirmed that alpha amylases had affinity for zeolites. The zeolite NaX resulted in 73% recovery of purified amylase. The results are encouraging for designing appropriate processes to recover this enzyme from fermentation broths.

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ANNEXURE

ANNEXURE

Figure A. Standard curve of amylase assay. Relationship between alpha amylase (as U/ml) and absorbance using starch-iodine assay, R^2 =0.85